Mutants of Pisum sativum (L.) altered in the symbiosis with Rhizobium leguminosarum.

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CHAPTER 7.

Summary and final conclusions

The studies on symbiotic nitrogen fixation between pea (Pisum sativum L.) and Rhizobium leguminosarum, which are described in this thesis, were carried out with the use of host mutants. Host mutants were obtained after mutagenic treatments of the seeds with ethyl methane sulphonate (EMS). Mutations were induced in genes which are directly or indirectly involved in the symbiosis. Using different types of host mutants, genetic, morphological-, biochemical- and physiological aspects of an altered symbiosis are described, always in comparison with a wildtype situation.

Because of the many different aspects of the symbiosis that have been studied, the thesis starts with an extended general introduction, which has been subdivided in four paragraphs (**Chapter 1**). In the first paragraph an overview is given of several symbiotic interactions existing between plants and nitrogen fixing micro organisms. The second paragraph describes the successive events in the interaction between legumes and rhizobia, starting with the excretion of (inducing) compounds in the root environment and finishing with a functional N_2-fixing root nodule. The third paragraph is about the presence and action of symbiosis specific proteins (or nodulins) and their corresponding host genes. The effect of mutations in bacterial symbiosis genes on host gene expression is briefly mentioned. In the fourth paragraph an overview is given of altered symbioses, which are the result of mutations or changes in the plant genome. Three classes of host mutants can be distinguished, featured by A. abundant nodulation associated with tolerance to nitrate (nts mutants) ; B. fixation deficiency (fix^- mutants) and C. nodulation resistance (nod^- mutants). Representatives of all classes were obtained before or during these studies.

Chapter 2 describes the selection of three symbiosis mutants in a M2 population of the cultivar Rondo. After backcrossing, the mutants have been compared in detail for morphology and nodulation properties with each other and with the wildtype Rondo. Mutant K5 was nodule free or weakly nodulating dependent on the culture conditions (root environment). Mutant K24 was nodule free under all tested growth conditions, while mutant nod3 was supernodulating and tolerant to nitrate, as had been shown before. Microscopic investigations showed characteristic symbiotic root hair curling on roots of K24, but infection threads were not seen (hac^+inf^-), in contrast with K5 (hac^+inf^+). All mutations were monogenic, recessive and non-allelic. Graft experiments showed that expression of the mutant characters was associated with the root genotype and in the case of K24 also with the shoot.

In **Chapter** 3 three series of nodulation resistant mutants with different genetic backgrounds (parent types) are described : mutants with wildtype cv. Rondo (K-series) and cv. Ascona (KA-series) and mutants with nod3 as supernod parent type (KN-series). A classification of the nod^- mutants was made based upon the absence or presence of symbiotic root hair curling, infection thread formation and nodulation behaviour in soil and hydroculture. It was found that mutants which had been selected in soil (KA-series) in general were nodule-free and apparently disturbed at an early stage in the interaction with Rhizobium ($hac^{-/+}inf^-$). In contrast, mutants selected in hydroculture (K- and KN-series) in majority were more or less nodulating depending on the growth conditions and either hac^+inf^- or hac^+inf^+.

Genetic experiments showed that the mutant characters were almost always monogenic and always recessive. Tests of allelism showed that, within the group of investigated mutants, most mutations were non-allelic and that no less than fourteen separate genes were directly or indirectly involved in nodulation. Graft experiments showed that the expression of mutated genes in chimeric plants was associated with the genotype of the root and only in a single case also with that of the shoot.

A description of the fix$^-$ mutant FN1 (nof1) is given in **Chapter 4**. In comparison with the parent type nod3 this monogenic recessive mutant has even more nodules and the average size of the ineffective nodules is higher than that of effective ones. Morphologically and anatomically the ineffective nodules were hardly different from effective ones. However, light- and electron microscopic studies showed that the bacteroids inside the cortex cells of the fix$^-$ mutant presumably were deteriorating sooner, as judged from the disorganized structures of the enclosing membranes. The storage of starch in the cortex cells once more indicated the disturbance of the nitrogen fixation metabolism.

In **Chapter 5** mutant FN1 has been analyzed at protein level. The ineffective nodules of this mutant were clearly less reddish than effective nodules and contained a lower content of leghemoglobin, the protein which regulates the oxygen tension within the nodule. All isoforms of leghemoglobin were present in fix$^-$ nodules, but the (in vitro) affinity for oxygen seemed to have changed, which could be a plausible reason for non-fixing. No additional demonstrable difference was found between the protein patterns of the cytosol of the fix$^-$ mutant and the parent type. All known nodulins were also found in the ineffective nodules. Nitrogenase was hardly detectable in the fix$^-$ bacteroids, but this was the only clear difference between the protein patterns of functional and non-functional bacteroids. Two 18 kDa proteins, which were abundantly present in the peribacteroid space between the cytosol and the bacteroid of the parent, were absent in mutant FN1. However, the relation between these proteins and the functioning of nitrogenase (and/or leghemoglobin) remained unclear.

Absence of nodules as well as the presence of non-functional nodules affect plant metabolism. Some aspects, like nodule enzyme activities, amino acid contents of the xylem sap and plant growth have been studied as described in **Chapter 6**. For this purpose the nod$^-$ mutant KN7 and the fix$^-$ mutant FN1 were compared with the parent type nod3. Dry matter production and nitrogen accumulation in non-fixing plants appeared to be higher than in nod$^-$ plants, although growth of both kinds of mutants was considerably retarded compared with that of parent type plants. Analyses showed that the xylemsap of parent type plants contained already a high content of amino acids after a relative short period of growth, in contrast with nod$^-$ and fix$^-$ plants. After a prolonged period of growth it was found that appreciable amounts of amino acids were translocated from the fix$^-$ nodules towards the shoots. This means that a nitrogen metabolism got started in these nodules, which might be the (combined) result of a low (in vivo) nitrogenase activity, a conversion of nitrate (present in the culture medium) into ammonium and deteriorating bacteroids. This agreed with the measured (in vitro) activities of the key enzymes GS, GOGAT and PEP carboxylase in fix$^-$ nodules, which were not different from those in effective nodules.

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The characterization of the mutants described in this thesis has resulted in a deeper understanding of the functions of legume host genes.
The effects of mutated host genes in general are pleiotropic. This was already shown for mutant nod3, having an abundant nodulation, a compact root system and ability to form nodules in the presence of nitrate. Pleiotropic effects were also found during studies on the fix- mutant FN1. Some of the observed differences with the parent type, like the abundant presence of starch granules in the root cortex cells, retarded growth and N-deficiency symptoms, almost certainly are the result of non-fixing. Likewise the near absence of the nitrogenase proteins in the bacteroids must be caused by a plant determined factor. Leghemoglobin malfunctioning and absence of two prominent proteins (if these are plant derived) in the PBS may be directly associated with the primary transcription product of the mutated gene and the cause of non-fixing. Differences in morphology of the root systems and root hairs of nod- mutants can also be considered to be the results of pleiotropic effects of the mutated genes.
Fourteen different genes have been found to be involved in (non-) nodulation. Some of them affect the root- and root hair morphology and non-nodulation may be a logical result, since absence and deformation of root hairs can prevent infection. Only part of the induced mutations probably are in symbiosis specific (nodulin-) genes.
The expression of the mutant characters was not always the same. On roots of many "nod-" mutants occasionally nodules were formed, depending on the growth conditions. Also the non-fixing feature of mutant FN1 may not be absolute, regarding the results on acetylene reduction activity measurements and the studies on nodule metabolism. For that reason several mutants with a low acetylene reduction activity were not included in this study. Nevertheless, in comparison, many more nod- mutants than fix- mutants could be selected which indicates that a relatively high number of host genes is involved in the formation of nodules and a low number is involved in the functioning of the latter.
Until now molecular studies on host gene expression in pea have been done on normal (fix+) symbioses and altered symbioses as result of bacterial mutations. For this purpose cDNA clones and specific antibodies raised against nodule proteins were used. Similar studies on host mutants will increase the knowledge of the relation between host genes and host gene products. Therefore it is an advantage that expression of the mutated genes in our study was always associated with the root and not with the shoot.
To accomplish the studies, isolated host genes can be transferred to related legume species (Stougaard et al., 1987) or to host mutants, using modern gene transformation methods. Hence, the expression of a single gene can be studied, while reparation of the host mutants by gene complementation becomes a possibility. With similar gene transformation techniques it might finally become possible to transfer host genes to non-leguminous plants in order to establish a symbiosis with nitrogen fixation organisms. The detection, in our study and those of others, of numerous different plant symbiosis genes already is an indication that this idea is wishful thinking for the moment. However, recent reports showed that nodule formation can simply be induced with hormone inhibitors in the absence of rhizobia (Hirsch et al., 1989), while rice roots became susceptible to infection with Rhizobium after a simple treatment with cell wall digesting enzymes (Al-Mallah et al., 1989). These examples suggest that much genetic information required for effective symbiosis may be present in non-legumes. The absence of a functional interaction between such plants and rhizobia may be the result of the missing of only a few key genes.